61 *Stachybotrys*

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CONTENTS

61.1 INTRODUCTION

61.1.1 Classification, Morphology, and Biology

The genus *Stachybotrys* is an asexually reproducing, dematiaceous fungus belonging to the mitosporic Hypocreales group, order Hypocreales, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. The mitosporic Hypocreales group encompasses the genera of *Acremonium*, *Acrostalagmus*, *Cephalosporium*, *Chaetopsina*, *Cylindrocladiella*, *Escovopsis*, *Fusarium*, *Gliocladiopsis*, *Gliocladium*, *Hobsonia*, *Illosporium*, *Myrothecium*, *Parasarcopodium*, *Polycephalomyces*, *Rotiferophthora*, *Sesquicillium*, *Solheimia*, *Stachybotrys*, *Stilbella*, *Trichothecium*, *Tubercularia*, *Ustilaginoideae*, *Verticillium*, and *Xenocylindrocladium* [1].

In turn, the genus *Stachybotrys* is divided into 15 recognized species: *Stachybotrys bisbyi*, *Stachybotrys chartarum*, *Stachybotrys chlorohalonata*, *Stachybotrys cylindrospora*, *Stachybotrys dichroa*, *Stachybotrys echinata*, *Stachybotrys elegans*, *Stachybotrys kampalensis*, *Stachybotrys longispora*, *Stachybotrys microspora*, *Stachybotrys nephrospora*, *Stachybotrys oenanthes*, *Stachybotrys parvispora*, *Stachybotrys subsimplex*, and *Stachybotrys theobromae*, in addition to 13 unassigned species. Two species, *S. chartarum* (obsolete synonyms: *S. alternans* and *S. atra*) and *S. echinata* (obsolete synonym: *Memnionella echinata*), are implicated in human diseases [2,3].

Stachybotrys spp. grow rapidly and mature in approximately 4 days. Colonies are "cottony," white initially and turning black with age. Septate hyphae are hyaline first and become darkly pigmented later. Conidiophores are simple or branched, bearing phialides at apices. Phialides are hyaline or pigmented and cylindrical in shape, with swollen upper portions, forming clusters of 3–61. Conidia (4.5 × 9 μm) are oval, hyaline or pigmented, one-celled, and in clusters.

First identified from a mold growing on domestic wallpaper in Prague in 1837, *Stachybotrys* is a member of the Fungi Imperfecti known as "black mold" or "toxic black mold." The fungus is an inhabitant of soil and strata rich in cellulose (e.g., hay, straw, grain, hemp, plant debris, dead roots, wood pulp, cotton, fabrics, paper, book bindery glue, plant fiber-processing plants, etc.). It has been isolated from contaminated grains, tobacco, insulator foams, indoor air, and water-damaged buildings. The fungus tolerates temperature up to >60°C and survives over winter. *Stachybotrys* spores stay viable for years to decades, and conidia retain viability after passage through the gastrointestinal tract. However, the organism is killed by composting degradation of manure and by disinfectants [4].

Stachybotrys chartarum is a known producer of trichothecene mycotoxins and stachylysin (a hemolysin). The best characterized trichothecenes include satratoxins F, G, and H, roriden E, verrucarin J, and trichoverrols A and B, which share chemical formula of $C_{25}H_{34}O_6$ or $C_{26}H_{38}O_6$ and are tricyclic sesquiterpenes with a 12,13-epoxy-trichothec-9-ene ring. *Stachybotrys* species also produce spirolactams and spirolactones (related to anticomplement components), phenylspirodrimanes (inhibitor of complement activation), cyclosporins, and endothelin receptor antagonists [5–7].

Trichothecenes modulate inflammatory reactions and alter alveolar surfactant phospholipid concentrations, besides being potent inhibitors of protein synthesis (e.g., scirpentriol, 15-acetoxyscirpendiol, diacetoxyscirpenol (DAS or anguidine), verucarin A, and T-2 toxin) and elongation or

termination (e.g., trichodermin, trichodermol, crotocol, trichothecolone, trichothecin, and verrucarol). They are susceptible to destruction by alkali although resistant to sunlight, UV light, x-rays, heat (up to 120°C), acids, and trichothecenes. The mycotoxins have been isolated from dust (e.g., satratoxins, trichoverrols, verrucarol, verrucarins, and trichoverrins) and grain (T-2 toxin, nivalenol, and derivatives of others). Humans may develop toxin-related disease by ingestion of food products contaminated with the fungus and/toxins, exposure to mycotoxins in building (sick-building syndrome), and/or inhalation of propogules while undertaking experiments with the fungus [8].

61.1.2 Clinical Features and Pathogenesis

61.1.2.1 Clinical Features

Stachybotrys may induce disease by (1) infection, (2) generation of a deleterious immune response, and (3) toxic-irritant effects from mold metabolites. Together with other molds (e.g., *Aspergillus*, *Penicillium*, *Alternaria*, and *Cladosporium*), *Stachybotrys* may play a role in the development of sick-building syndrome [9–25].

Stachybotrys was first noted as a pathogen of horses in Ukraine in the early 1930s. After the ingestion of hay contaminated with *Stachybotrys*, horses developed lip edema, stomatitis, oral necrosis, rhinitis, conjunctivitis, coagulopathy, hemorrhage, and neurologic disorders (irritability, gait disturbance, and blindness). So-called "superinfections" occurred and deaths were observed. The disease syndrome is referred to as stachybotrytoxicosis. In a rare "atypical" or "shocking" form, the disease was primarily neurological and highly fatal, with areflexia (loss of sensorimotor reflexes), hyperesthesia (hypersensitivity to pain), hyperirritability, blindness, and stupor. Cattle were also affected to a lesser extent, and younger animals fared better than older one. Similar diseases have been reported in other parts of the world. A case of sheep disease was described in the 1990s after animals consumed heavily contaminated grain cubes in South Africa. The affected animals displayed fever, listlessness, oral lesions, pancytopenia, hemorrhage, opportunistic infections, and a significant mortality rate [4,26–28].

In areas of enzootic equine stachybotrytoxicosis, fodder handlers and others with close contact with musty straw (e.g., using straw for fuel or bedding) also developed a dermatologic and respiratory syndrome. Dermatologic symptoms were dermatitis on the scrotum, medial thighs, axilla, the hands, and other areas, which progressed from hyperemia to crusting exudates to necrosis, with subsequent resolution. Some patients showed erosions on the oral and gingival mucosa. Respiratory symptoms included catarrhal angina, bloody rhinitis, cough, throat pain, chest tightness, and occasional fever. Some patients had transient leukocytopenia (reduced white blood cell count) and hemorrhage (bleeding). *S. chartarum* (*S. alternans*) isolated from straw produced areal fructifications in experimental rabbits by a dermal toxicity test and similar local and systemic responses on the skin of volunteers [4].

Since then, other clinical manifestations have been associated with exposure to *Stachybotrys chartarum* mycotoxins and spores, ranging from (1) chronic fatigue or headaches, (2) fever, (3) irritation to the eyes, mucous membranes of the mouth, nose, and throat, (4) sneezing, (5) rashes, (6) chronic coughing, (7) nausea, (8) memory loss, (9) vomiting, (10) bleeding in the lungs and nose, (11) hypersensitivity pneumonitis (HP), (12) allergic rhinitis, and (13) asthma exacerbations. Occupants of mold-contaminated, water-damaged buildings often develop symptoms in the central nervous system (CNS) and the immune system as well as pulmonary diseases, allergy, and inflammatory reactions. Occupational stachybotrytoxicosis acquired by inhalation showed chest and upper airway symptoms, fever, leucopenia, and dermatitis, which started within 2–3 days of exposure and lasted for 3 weeks [18]. In a recent investigation of 32 patients with symptoms attributed to mold exposures at work, 25 (88%) patients nominated *S. chartarum* as well as *Aspergillus* and *Penicillium*. 79%, 70%, and 64% of the 32 patients presented with cough, shortness of breath, and chest tightness, respectively, which persisted more than 6 weeks in 91%, suggestive of sick-building syndrome. Thirty one percent of these patients had positive skin test to fungal extracts, suggesting IgE-mediated or other nonimmune mechanisms could be the cause of their symptoms [25,29].

61.1.2.2 Pathogenesis

Contemporary construction methods that use cellulose substrates (e.g., fiber board) favor the growth of cellulolytic fungi such as *S. chartarum* (Meggs [23]). The fungus is shown to produce trichothecene mycotoxins as mentioned above, although several other fungi (e.g., *Fusarium*, *M. verrucaria*, *M. roridum*, *Trichothecium*, *Trichoderma*, *Cephalosporium*, *Verticimonosporum*, and *Cylindrocarpon*) also synthesize these compounds. *S. chartarum* macrocyclic trichothecene mycotoxins (MTM) are dissociated readily from the surface of the organism and are consequently spread in damp buildings. *S. chartarum* MTM remain toxic over extended periods of time, and individuals with exposure to the fungus contain MTM in their sera [24]. High indoor exposures to trichothecene mycotoxins are associated with infrequent ventilation or vacuuming of the building, pets, visible mold, and old carpets [18].

Trichothecenes are potent translational inhibitors and stress kinase activators (Pestka et al. [48]). Experimental exposure of trichothecene mycotoxins in mice led to severe intra-alveolar, bronchiolar, and interstitial inflammation. In experimental Wistar rats, decreased alveolar macrophages viability and increased activity of the lysosomal enzyme cathepsin D in bronchoalveolar lavage cells after *S. chartarum* exometabolite exposure are noticeable [30]. *S. chartarum* metabolites suppress red blood cell (RBC); decrease the total RBC count, hemoglobin, and hematocrit; and increase total bronchoalveolar lavage fluid cell count (indicating inflammation, lower alveolar macrophage counts, and increased granulocyte count related to the BALF cells) in these animals [31].

Satratoxin-positive *S. chartarum* activates inflammasome-associated caspase-1, which is needed for proteolytic processing of IL-1beta and IL-18, in human macrophages. In addition, purified trichothecene mycotoxins, roridin A, verrucarin A, and T-2 toxin activate caspase-1 and strongly enhance LPS-dependent secretion of IL-1beta and IL-18. Satratoxin-positive *S. chartarum* and the trichothecenes it produces also trigger the activation of caspase-3, which is an effector caspase of apoptosis. Thus, human macrophages sense trichothecene mycotoxins as a danger signal, which activates caspase-1 and further enables the secretion of IL-1beta and IL-18 from the LPS-primed cells [32].

Several enzymes from *S. chartarum* spores demonstrate proteolytic activity and are able to hydrolyze gelatin and collagen I and IV [33]. *S. chartarum* spore extracts induce high levels of IL-6, IL-8, and TNF-alpha in human tracheal epithelial cells. This stimulation of cytokine production is abolished by a serine protease inhibitor Pefabloc. Thus, proteinases from *S. chartarum* spores significantly contribute to lung inflammation and injury [34]. *S. chartarum* spores are capable of inducing both apoptosis and necrosis in THP-1 cells, leading to cell death within 3–6 h. More specifically, *S. chartarum* spores increase the formation of reactive oxygen species (ROS) and oxidative DNA damage. *S. chartarum* trichothecenes T-2 toxin and satratoxin G are mainly responsible for apoptosis [35].

61.1.3 Diagnosis

S. chartarum is a cellulose-decaying fungus that grows well at room temperature and with humidity above 93%. Isolation of *S. chatarum* requires special media with high concentration of cellulose and low concentration of sugar and nitrogen to compete with *Penicillium* and *Aspergillus*. Growing *S. chatarum* isolates on rice or potato dextrose agar results in higher proteolytic activity of the spores than those grown on drywall.

Thin-layer chromatography, high-performance liquid chromatography (HPLC), tandem mass spectrometry (MSMS), gas chromatography (GC), GC combined with mass spectrometry (GC-MS, GC-MSMS), and enzyme-linked immunosorbent assay (ELISA) have been applied to detect *S. chartarum* mycotoxins in mold-affected materials inside buildings, in carpet dust from water-damaged buildings, and in animal tissues [36–41]. Finally, various reports have demonstrated the potential usefulness of molecular biological techniques for detection of molds in air and clinical specimens [42–45].

61.2 METHODS

61.2.1 Sample Preparation

61.2.1.1 Trichothecene Collection and Analysis

Airborne trichothecene mycotoxins are collected using a SpinCon PAS 450-10 bioaerosol sampler (Sceptor Industries) and an Andersen GPS-1 polyurethane foam (PUF) high-volume air sampler (Thermo Electron Corporation). Entrained solids are concentrated in a phosphate-buffered saline (PBS) solution (pH 7.4) to a final volume of 10 mL [46].

Following the collection, SpinCon samples (all 10 mL) are filtered using Fisher 13 mm-diameter nylon syringe filters with a 0.45 µm pore size (Fisher Scientific). The filtered fluid is transferred asceptically to 15 mL polypropylene conical centrifuge tubes, frozen at −80°C, and lyophilized using a VirTis Freezmobile (SP Industries). The dried samples are individually resuspended in 1 mL of pyrogen-free water (25°C) for immediate testing.

Filters obtained from the Andersen PUF sampler are transferred individually to 50 mL polypropylene centrifuge tubes on-site. The filters are suspended in 40 mL of PBS, vortexed vigorously for 60 s, removed from the tubes using sterile forceps, and then discarded. The PBS extracts are filtered into new 50 mL tubes. These are frozen at −80°C, lyophilized, and resuspended in 1 mL pyrogen-free water for immediate testing.

Samples are analyzed for macrocyclic trichothecenes using a QuantiTox kit for trichothecenes (EnviroLogix). This competitive ELISA kit incorporates trichothecene-specific antibodies immobilized in polystyrene microtiter wells and is highly specific for MTM of *S. chartarum*. To ensure that the ELISA ran correctly, the macrocyclic trichothecene roridin A is used at a concentration of 50 ng/mL in PBS as a positive control for each set of tests. PBS alone is used as a negative control [39].

Conidia and other airborne particulates are collected on glass microscope slides that have been coated with a thin layer of petroleum grease using a foam makeup applicator. For testing purposes, conidia are collected from plates that have reached confluence (approximately 7–14 days), using sterile cotton swabs. To collect the conidia, swabs are gently rolled over the surface of the fungal growth. The cotton tips of the swabs are placed in 1 mL of sterile room-temperature (25°C) PBS in 1.5 mL microcentrifuge tubes and vortexed for approximately 1 min to remove conidia. The conidia are then counted using a hemacytometer and identified to the genus level by a trained technician using an Olympus BH2-RFCA optical light microscope. For ELISA testing, the fungal spore suspensions are centrifuged at 14,500 rpm for 1 min to pellet the conidia. Care is taken not to disturb the conidium pellet, and only the top 80% of the supernatant is used for ELISA testing. Each sample is run in duplicate wells on two separate occasions [39].

61.2.2 Detection Procedures

Pounder et al. [45] described a real-time PCR with SYBR green DNA-binding dye and amplicon-melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′). The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. The PCR mixture is composed of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$ (additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 µM each of ITS1 forward and ITS4 reverse primers, 1× SYBR green (Molecular Probes), and 3 µL template DNA.
2. Thermal cycling parameters are 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45-s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 µL of each primer (0.8 pmol/µL) and 3 µL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note: Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%. For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit rRNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification [47].

61.3 CONCLUSION

The genus *Stachybotrys* consists of large number of cellulose-decaying fungal species that are distributed in soil, plants, air, and water-damaged buildings [48]. These fungi produce several mycotoxins that are potent translational inhibitors and stress kinase activators, leading to diseases in humans. Along with biochemical procedures, molecular techniques have been developed to detect the fungus and its mycotoxins [49].

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AUTHOR QUERIES

[AQ1] Please verify the edit made to the sentence beginning "They are susceptible to…"

[AQ2] Please check if the removal of the open parenthesis in the sentence beginning "The PCR mixture is composed of 1× Lightcycler …" is ok.

[AQ3] Please provide accessed date for Ref. [1].